3.) BEST AVAILABLE COPY

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Setting up Allo- & Self restricted peptide CTL Lines

PBMC: LG3 A2⁻ Allo

LG4 A2+ self

peptides: WT1 10, 235, 441 and C228 (+ve Control).

Method: as left — use DC from LG4 as stimulator

Restimulate with peptide Loaded C1RA2 cells. (50 nM)

Restimulated c " RMA-S-A2 (50 nM).

"

CTL assay

Expand & cloning

Sets of clones

Transfer the well to 48 well plate

Feed

5

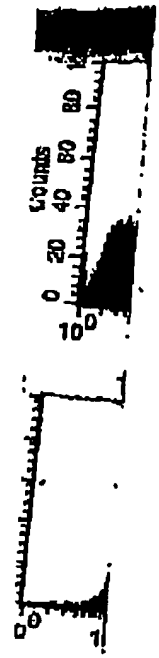
Setting up: Self & Allo-restrict peptide specific CTL line

PBMC LG5 A2+ Self
LG6 A2+ Allo

peptides: WT-1 126. 187. 280
+ve control E786-9V.

Methods, see previous page.
First round Stimulation with T2 loaded peptides.
Restimulate with peptide loaded-CIR A2
1/2 RMA-S-A2
3/4 Restimulation CIR A2
2nd
7/3 CTL
2nd 3 Cloning
14th CTL of clones

16



Protocol for peptide specific allorestricted human CTL

1. Prepare stimulator cells: in case of T2 stimulators preincubate o/n 10^6 cells/ml/well in x24 well plates in RPMI medium plus 5% boiled (10 min) FCS with 100 uM peptide. If allo Dc are used as stimulators, allo DC from A2 +ve individuals can be separated as following:

Incubate A2+ PBMC at 37°C O/N, harvest nonadherent cells, resuspend in RPMI+9%FCS at $5X10^6$ /ml and layer onto metrizadime gradient carefully, spin at 1500 for 10 min and harvest the interface (30% low density DC, 20% recovered from PBMC counts). Wash X3 with medium, resuspend at 10 ^{6}/ml, add pepdides to a final conc of 50uM and incubate at 37°C for 4hr. (3000 rad) The stimulator can be added to responders directly w/O washing off peptides.

2. Separate blood by Ficoll centrifugation, wash twice, count, make sure that cells are HLA-A2 negative (by staining with HB54 and HB117 antibodies)

3. Plate $2x10^6$ effectors and $2x10^5$ stimulators per well in x24 well plates in culture medium (RPMI 1640, 10% FCS, 1% glutamine, 1% pen/strept $5x10^{-5}$ M 2-ME) with 500 nM peptide

4. On day 4 prepare stimulators as described in 1. if required. It may be not necessary to preincubate C1RA2 cells for more than 2 hrs

5. On day 5 harvest cells, count spin and seed $5x10^5$ effectors, $2x10^6$ irradiated (about 3000 rad) HLA-A2 negative autologous feeders, $2x10^5$ pepride loaded and irradiated (about 10000 rad) T2 or C1RA2 cells and 500 nM peptide in 2ml of the culture medium with 10u/ml IL-2, 2.5ng/ml IL7 (1/2000 of the stock of 5ug/ml) and with 10% of Q12054 culture sup (anti-CD4 antibodies) (complete medium)

6. 14 days (and further every 2 weeks) later restimulate cultures as described in 5. Cell recovery is usually 200%

7. On day 5 analyse peptide specificity in a ^{51}Cr release assay using T2 or mouse cells transfected with A2 molecule as CTL targets and cold K562 cells as NK targets (10 per 1 labelled target). Use 0.7% TFA for max release instead of 0.5% SDS. Significant peptide specificity is detectable after 5-7 week in culture with E:T ratio 10:1

8. Peptide specific cultures can be cloned seeding 1, 10 and 100 cells/well in round bottom x96 well plates in complete culture medium with 10^4